Volume 177, number 2 FEBS 1985 November 1984

Lysosomal thyroid hormone 5′-deiodinase

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Received 23 August 1984

Lysosomes prepared from rat liver and kidney after loading with the detergent Triton WR-1339 show membrane-bound 5′-deiodinase activity with marked specificity for 3,3′,5′-triiodothyronine (reverse T3), lesser activity with respect to thyroxine (T4) and almost none towards 3,3′,5-triiodothyronine (T3). The enzyme is thiol dependent and shows maximal catalysis at pH 7.2. As many of the states known to alter thyroid hormone levels also affect lysosomal function, inhibition of the lysosomal 5′-deiodinase leading to an increase in intracellular reverse T3 may be an initiating mechanism for thyroid hormone change.

Lysosome *Deiodination* *Iodothyronine* *Detergent* *Dithiothreitol* *Membrane*

1. INTRODUCTION

In the low T3 syndrome, the thyroid hormone changes of lowered T3 and raised rT3 may be a homeostatic response to stress states in, for example, starvation, illness, and surgery, but the function of these changes is unknown. Further, the subcellular site(s) and control mechanism(s) for the 5′-deiodinase enzymes are controversial. In rats, 5′-deiodinase activity has been localised in the kidney to plasma membrane (PM) [1], and in the liver either to the endoplasmic reticulum alone (ER) [2,3] or to both the ER and PM [4].

In our studies of the intracellular sites for 5′-deiodination of thyroid hormones, we noted substantial 5′-deiodinase activity in the crude mitochondrial fraction which includes lysosomes. We therefore separated lysosomes from mitochondria and peroxisomes [5,6] and tested them for 5′-deiodinase activity.

2. MATERIALS AND METHODS

Protein determinations [7] were made in duplicate using bovine serum albumin (Sigma) as standard. Marker enzymes were determined in duplicate as follows: Glucose-6-phosphatase (EC 3.1.3.9) and 5′-nucleotidase (EC 3.1.3.5) were assayed [8] and liberated phosphate measured [9]. Succinic dehydrogenase (EC 1.3.99.1) was assayed by combining 0.1 ml of cell fraction with 0.5 ml of 0.2 M Na_2HPO_4 (pH 7.4), plus 0.3 ml of 0.2 M sodium succinate (pH 7.4), plus 0.1 ml of 1% (w/v) 3-(4,5-dimethylthiazole)-2,5-diphenyltetrazolium bromide (MTT, Sigma). Following incubation at 37°C for 15 min, the reaction was stopped by the addition of 1 ml of 7% (w/v) trichloroacetic acid and the colour extracted into 4 ml tetrahydrofuran then read at 550 nm. β-Glucuronidase (EC 3.2.1.31) was assayed using *p*-nitrophenol glucuronide (Calbiochem) [10]. *N*-Acetyl-β-glucosaminidase was assayed [11] using 4-methylumbelliferone derivative (Boehringer).

Lysosomes ('tritosomes') were isolated from rat livers [5,6] after the prior daily intra-peritoneal injection of Triton WR-1339 (50 mg/100 g) for 4 days, with the following modifications. A tissue press (Harvard) was used initially followed by 8 passes in a hand-held glass Dounce homogeniser, the homogenising buffer was expanded to contain sucrose, Tris–HCl, KCl, EDTA, dithiothreitol (DTT), chloroquine, 250:25:100:15:1:0.02 mM (pH 7.4). The need for a thiol cofactor was tested

Part of this investigation was presented to the 1984 annual meeting of the Endocrine Society of Australia

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

by omitting DTT from the homogenising buffer.

Tritosomal membranes were fractionated by 1:6 dilution of tritosomes with 25 mM Tris–HCl (pH 7.2) followed by immersion in liquid nitrogen and then rapid thawing by shaking at 37°C to lyse the membranes. After thawing, the tritosomes were brought to 4°C (for all succeeding operations) and centrifuged at 260000 × *g* (Beckman 70 Ti rotor) for 30 min and the supernatant decanted. The sediment was resuspended in 30 ml of 1.0 M KCl for 30 min and centrifuged and decanted as above. The remaining sediment was resuspended in 3.0 ml of 0.1% (v/v) Triton X-100 for 30 min and then diluted to 0.01% and again centrifuged and decanted as above. Each of the 3 supernatants and the final sediment were assayed (after storage for up to 14 days at −20°C) for both rT3 5′-deiodinase and *N*-acetyl-*β*-glucosaminidase in 0.01% (v/v) Triton X-100.

Enriched PM fractions were prepared from the livers of 200-g male rats [12]. The method was extended by retaining the upper layer after sedimentation of PM at 7000 × *g*. This latter layer was diluted to 250 nM sucrose and a crude mitochondrial fraction (12000 × *g* for 30 min) was sedimented. The supernatant was then centrifuged at 260000 × *g* for 60 min to give an enriched ER fraction.

Outer ring (5′-) deiodination by fractions at pH 7.2 containing DTT was semi-quantitatively estimated in duplicate by measuring [13] the enzymic release of $^{125}I^-$ from the following iodothyronines labelled in the outer ring (final assay concentrations in parentheses): T4 (100 nM), T3 (10 nM) and rT3 (10 nM). Radioisotopes were products of New England Nuclear. HPLC was used to separate and identify iodothyronines [13] modified to use 3′-isopropyl-3,5-diiodothyronine (a gift of the late Professor Jorgensen) as an internal standard. This iodothyronine migrates between rT3 and T4 in the chromatogram. Determinations of $^{125}I^-$ (by ion exchange) and then of iodothyronines (by HPLC) can be made on the same sample.

Quantitative kinetic analysis of 5′-deiodinase activities in cell fractions using the above techniques was done by warming a sample of enzyme at 37°C for 5 min and then adding to a variable substrate concentration spiked with a known quantity of tracer. The final concentration of DTT was 10 mM. At varying intervals (0–20 min) for rT3 and (0–60 min) for T3 and T4, duplicate samples of 0.5 ml were removed and the reaction stopped by addition to 0.5 ml of 7% (w/v) trichloroacetic acid. Initial velocities were determined by drawing tangents to the curves at the origin. Apparent maximum velocities (V_{max}) and Michaelis constants (K_m) were then determined [14].

3. RESULTS

Crude mitochondrial fractions and the subsequently isolated tritosomes (fig.1) exhibited both elevated lysosomal marker enzymes *β*-glucuronidase and *N*-acetyl-*β*-glucosaminidase and elevated rT3 5′-deiodinase with lesser levels of T4 5′-deiodinase. Similar results (not shown) were obtained for kidney tritosomes but protein yields were much less.

Variation in DTT concentration showed an obligatory requirement of the rT3 5′-deiodinase for DTT with maximal activity at approx. 10 mM for tritosomes from both liver and kidney.

The effect of pH on liver tritosomes was examined using phosphate, Tris and Hepes buffers. Reverse T3 5′-deiodinase has a sharp maximum at pH 7.2 in all 3 buffers. T4 5′-deiodinase has a broader maximum.

Fractionation of tritosomal membranes (table 1) showed that the rT3 5′-deiodinase enzyme is particulate and present as an integral protein in the lysosomal membrane requiring the use of Triton X-100 to be solubilised.

Analysis of the tritosomal rT3 deiodinase reaction, using parallel measurement of $^{125}I^-$ and iodothyronines indicated that I^- and 3,3′-T2 were, within the limits of the assay, formed in equimolar amounts. Similar analysis using T4 was not possible because of insufficient deiodination for analysis by HPLC.

Although concentrations of T4 (100 nM), T3 (10 nM) and rT3 (10 nM) and the time of incubation were selected to assess physiologically relevant 5′-deiodinase activity in the various subcellular fractions, such conditions give neither maximal velocities, nor linear velocities during incubation and are at best only semi-quantitative. Quantitative kinetic analysis of 5′-deiodination was made for T4, T3 and rT3 in the presence of saturating concentrations of DTT (10 mM) using

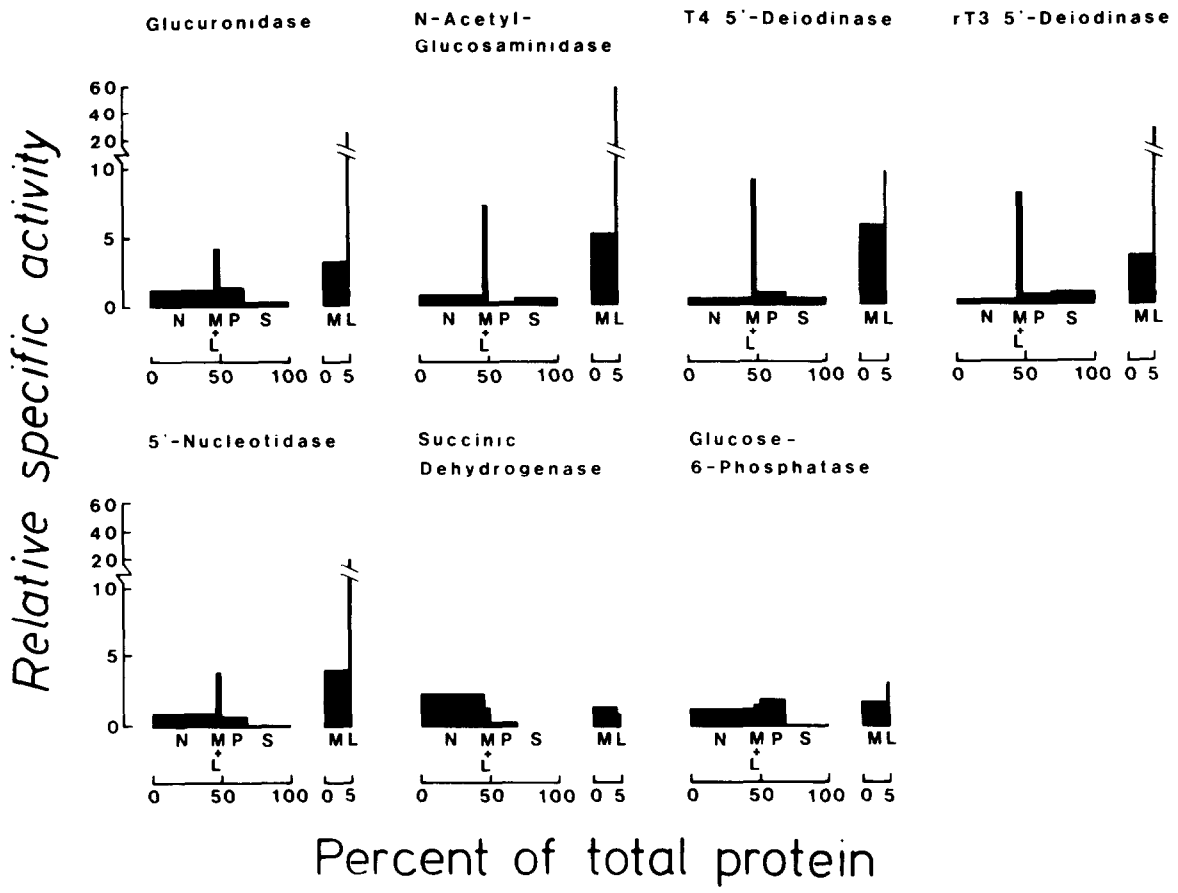

Fig.1. Marker enzyme and 5′-deiodinase activities were determined in liver cell fractions after prior injection of Triton WR-1339 [5]. The crude mitochondrial fraction (M + L) was further separated into lysosomal (tritosomal) and mitochondrial fractions [6]. The latter data are shown in the separate segment on the right-hand side of each panel. The data are expressed as specific activity relative to homogenate (ordinate) against percent of total protein in each fraction (abscissa). N, nuclei/plasma membrane fraction; M, mitochondria; L, lysosomes; P, microsomes; S, cytosol.

Table 1

Membrane localisation of tritosomal rT3 5′-deiodinase

Fraction	rT3 5′-deiodinase		*N*-Acetyl-β-glucosaminidase	
	Liver	Kidney	Liver	Kidney
Tritosomes	100	100	100	100
Tritosomes after freeze-thawing	135	117	108	128
Freeze-thawed supernatant 1	10	7	60	43
KCl supernatant 2	6	19	23	23
Triton X-100 supernatant 3	41	84	6	16
Triton X-100 sediment	14	13	2	10

Tritosomal membranes were fractionated as described in section 2. Triton X-100 0.1% (v/v) was added to each fraction to solubilise membranes but diluted to 0.01% (v/v) for assay. Enzyme activities are expressed as a mean percentage of the total value for freshly prepared tritosomes

Table 2

Kinetic parameters for iodothyronine 5′-deiodinase of fractions from rat liver

Parameter	Substrate	Tritosomes	Enriched plasma membranes	Enriched endoplasma reticulum
Michaelis constant	rT3	0.53	0.22	0.57
(K_m) (μM)	T4	0.50	0.24	1.90
Maximum velocity				
(V_{max})	rT3	209	42	313
(pmol/min per mg)	T4	56	8	38

Cell fractions were prepared and their 5′-deiodinase activities determined as described in section 2 in the presence of 0.01% (v/v) Triton X-100

tritosomes (table 2) and for comparison, enriched liver PM and ER from matched rats not injected with Triton WR-1339. Deiodination of T3 was not detectable with any fraction. Table 2 shows that there is a very active rT3 5′-deiodinase and a less active T4 5′-deiodinase in both the tritosomal and ER fractions, with much less activity in the PM fraction.

The PM and ER fractions derived from the same homogenate show good separation of the marker enzymes 5′-nucleotidase (PM) and glucose-6-phosphatase (ER). When assayed in the absence of Triton X-100 both fractions show T4 5′-deiodinase activity with PM being more active (fig.2). Thus both the degree of solubilisation and the presence of detergent is important in determining the apparent rates of reaction.

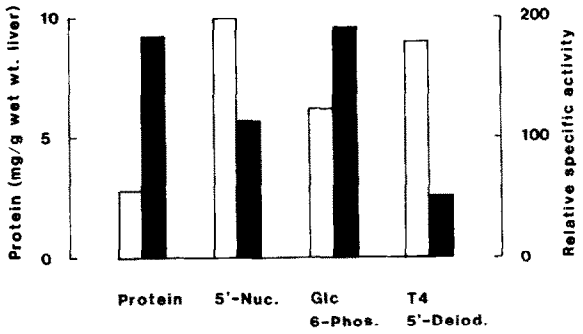

Fig.2. Comparison of marker enzymes from enriched plasma membrane (open bars) and enriched endoplasmic reticulum fractions (closed bars). The enriched fractions were prepared from rat liver by modifying [12] (see section 2). 5′-Nuc., 5′-nucleotidase; Glc 6-phos., glucose 6-phosphate; T4 5′-deiod., T4 5′-deiodinase.

4. DISCUSSION

The cell fractionation data suggest that little or no 5′-deiodinase was associated with mitochondria as such. Rather, the concurrence of lysosomal marker enzymes with rT3 5′-deiodinase activity in tritosomes together with its tight membrane binding suggests very strongly that the enzyme is an integral part of the lysosome. Although the majority of lysosomal enzymes are soluble and occur in the matrix, a number have been shown to occur as part of the lysosomal membrane [15,16]. The tightly bound membrane site for rT3 5′-deiodinase implies that it is not the source of the soluble 5′-deiodinase which has been found in variable yield in the cytosol by [13,17]. To improve the separation of unbroken tritosomes, chloroquine was added to the homogenising buffer [18].

Whilst the level of 5′-nucleotidase in the tritosomes might be thought to suggest a plasma membrane contamination, this does not appear to be likely for three reasons: (a) the most highly purified preparation of lysosomes yet reported show significant enrichment of 5′-nucleotidase [19], suggesting that it has a dual lysosomal and PM location; (b) the specific activity of the PM 5′-deiodinase is less than that of the tritosomes suggesting that membrane traffic from PM to lysosome is not the explanation; (c) the fractionation studies in which the tritosomal 5′-deiodinase activity could not be removed by freeze-thawing or

by strong salt solution suggest that it is indeed an integral protein of the lysosomal membrane.

A definite lysosomal 5′-deiodinase enzyme activity has not previously been demonstrated. It has been suggested [2] that tritosomes may possess a 5′-deiodinase. However, the substrate used was T4 and not rT3, thus the presence of a highly active rT3 5′-deiodinase remained undetected. Here, it is evident that the lysosomal 5′-deiodinase activity is much more specific for rT3 than for T4, with virtually no activity towards T3. It is similar to other described 5′-deiodinases (review [20]) in having a near to neutral pH optimum at 7.2 and requiring a thiol cofactor.

The analysis of enzyme kinetics is based on the assumption that both the enzyme and reactants are in true solution. This is clearly not so for particulate enzymes and probably not so for substrates, such as thyroid hormones, which are poorly water soluble and thus usually protein bound in aqueous solutions. For a more valid comparison of the particulate deiodinase enzymes from various subcellular sites, they were initially solubilised in Triton X-100 followed by dilution to reduce any inhibitory action of this detergent. In fact, solubilisation appears to increase markedly the 5′-deiodinase activity of the ER relative to the PM fraction when compared to the assay of the particulate enzymes without Triton X-100. Latency of membranous enzymes is well known [8]. Thus both the degree of solubilisation and the presence of detergent are important in determining apparent rates of reaction.

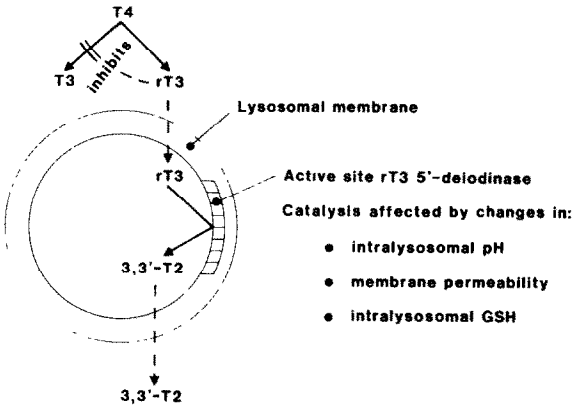

Fig.3. A model for the lysosomal control of triiodothyronine metabolism. The model shows the factors which could potentially alter the catalytic rate of lysosomal rT3 5′-deiodinase and hence alter the intracellular pool of rT3. As the concentration of rT3 varies, it will in turn lead to a varying inhibition of the enzymic production of T3 from T4 within the cell.

The finding of a highly specific lysosomal rT3 5′-deiodinase suggests a new model for the control of iodothyronine metabolism. This model (fig.3) suggests that inhibition of the lysosomal 5′-deiodinase, for example, by the fall in intralysosomal pH during autophagy, will lead to an increase in the intracellular concentration of rT3 which in turn will inhibit the production of T3 from T4 which has been shown in vitro by [21]. The model leads to the following predictions.

(i) Reverse T3 levels will change prior to changes in T3 levels. This was observed in vivo [22,23] in thyroidectomised and normal people respectively.

(ii) In the low T3, elevated rT3 state ('low T3 syndrome'), a decrease in the rate of breakdown of rT3 and a decrease in the rate of synthesis of T3 will occur. This is in good agreement with various kinetic studies performed in man during the low T3 syndrome (review [20]).

(iii) A number of drugs known to induce the low T3 syndrome may do so in part by their lysosomotrophic action, for example dexamethasone, propranolol and salicylate which are known to stabilise the lysosomal membrane [24].

Thus the model leads to a number of testable predictions regarding lysosomal function and thyroid hormone metabolism.

ACKNOWLEDGEMENT

The work was supported by a University of Tasmania Research Grant.

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